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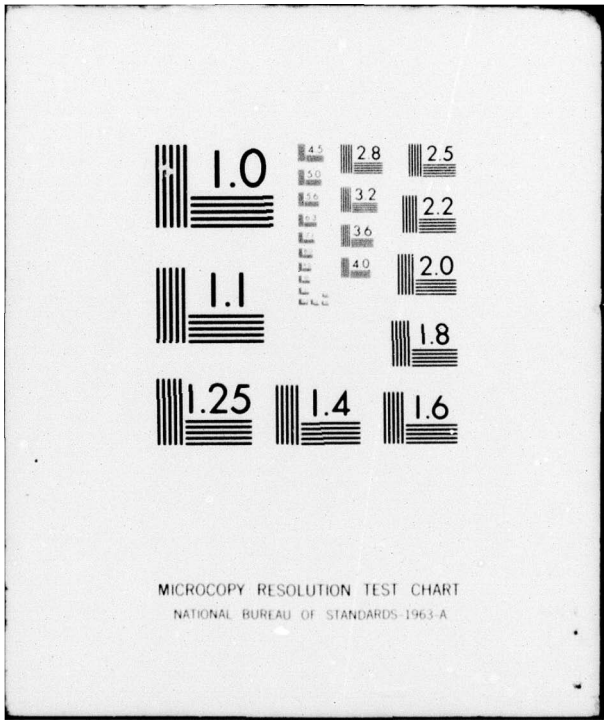
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Susumu Ito, Ph. D.

Annual Progress Report
(1 January 1976 - 31 July 1977)

Report Date October 25, 1977

Supported By
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This study is directed towards the isolation of rickettsial antigens and detection of antibodies. Stock supplies of Gilliam, Kato and Karp strains of Rickettsia tsutsugamushi were propagated in embryonated chick eggs and used for infecting cultured cells. Infected McCoy cell extracts containing soluble antigen as well as infected yolk sacs were inoculated into guinea pigs to produce antisera. However the yield of antibody was low and only detectable by fluorescent antibody techniques. More recently progress has made in growing →

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R. tsutsugamushi in BHK21 cells as a monolayer or in suspension cultures. Irradiated cells seem to enhance rapid rickettsial proliferation. Solubilization after freezing and thawing and by sonication and Triton X-100 treatment was used to isolate soluble antigen for characterization and further purification.

An enzyme immunoassay test to detect antibodies to Rochalimeae quintana in sera from patients with trench fever was evaluated with soluble antigens. This test detected antibodies in sera from known cases that were negative to other serological tests. However, there is an antigen that cross reacts with other rickettsia which appears to be a lipopolysaccharide antigen. This lipopolysaccharide seems to be related serologically to scrub typhus antigen.

In these studies infected cells as well as isolated samples used in the studies were fixed and examined by electron microscopy to monitor the structure of the organism and the host cell as well as some of the isolated and purified samples.

Summary

This study is directed towards the isolation of rickettsial antigens and detection of antibodies. Stock supplies of Gilliam, Kato and Karp strains of Rickettsia tsutsugamushi were propagated in embryonated chick eggs and used for infecting cultured cells. Infected McCoy cell extracts containing soluble antigen as well as infected yolk sacs were inoculated into guinea pigs to produce antisera. However the yield of antibody was low and only detectable by fluorescent antibody techniques. More recently progress was made in growing R. tsutsugamushi in BHK_{21} cells as a monolayer or in suspension cultures. Irradiated cells seem to enhance rapid rickettsial proliferation. Solubilization after freezing and thawing and by sonication and Triton X-100 treatment was used to isolate soluble antigen for characterization and further purification.

An enzyme immunoassay test to detect antibodies to Rochalimeae quintana in sera from patients with trench fever was evaluated with soluble antigens. This test detected antibodies in sera from known cases that were negative to other serological tests. However, there is an antigen that cross reacts with other rickettsia which appears to be a lipopolysacchardie antigen. This lipopolysaccharide seems to be related serologically to scrub typhus antigen.

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Foreward

This Annual Report covers studies undertaken or in progress on rickettsial diseases and supported by the US Army Medical Research and Development Command. These undertakings are a continuation of an ongoing project which has been directed towards the isolation and purification of rickettsial antigens and the detection of rickettsial antibodies of scrub typhus and trench fever.

In raising antibodies, withdrawing antisera, and performing toxicity tests the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

Report

Personnel: The changes which have occurred during the past year have been marked and most of these have been detailed in the various letters and reports that we have made to this granting agency. Since July 1, 1976 Dr. J. William Vinson has been retired from active academic duties in the Department of Microbiology. However, he was still retained and on salary until January 1, 1977. He still frequently visited the laboratory and helped us by advising us on many aspects of rickettsial cultivation and related serological studies. During the coming year, he has agreed to continue his interest in our work and will collaborate with us in some aspects of the proposed studies.

Dr. Jack Whitescarver who shared the major daily responsibility of managing his program in rickettsial studies has resigned his research associate position to take on an administrative post at NIH on June 20, 1977. His loss to the program put the present study in a precarious position but fortunately, Dr. A. Bruce MacDonald who was already a Professional Assistant on this Research Contract became available and interested in taking on a major responsibility in this project. In the relatively short period of just over two months, Dr. MacDonald and his research assistant have made rather remarkable strides in the cultivation as well as purification of antigens from Rickettsia tsutsugamushi. Some of these early results are elaborated in various parts of this proposal. The enthusiasm and vigor of Dr. MacDonald's approach to the rickettsial antigen problem seems to be showing considerable promise. Since Dr. MacDonald will be the primary person to be involved in the execution of a major portion of this proposal, it is appropriate that he is the other principal investigator. The background and closely parallel study that Dr. MacDonald has and is doing with chlamydia seems to fit in very well with the proposed rickettsial studies. Many of the same techniques can be transferred directly to the present work and so far these techniques seem to be ideally suited for these studies.

Dr. Ito and Dr. Vinson continue their close collaboration in their ultrastructural studies and the current collaboration of the ultrastructural analysis of the rickettsial cultures has again proven to be a valuable and complimentary check on the identification, proliferation, and control of contamination by bacteria, mycoplasma and viruses.

In the past month there has been a major administrative change in the Department of Microbiology. Professor Roger L. Nichols has given up the chairmanship and Dr. Jan Cerny has accepted the acting chairmanship. Dr. Cerny has heartily endorsed the continuation of the present studies and will support its activities with allocation of adequate space and facilities. The Dean of the School of Public Health, Dr. Howard Hiatt has also been consulted and supports this ongoing project. With these favorable endorsements, it is expected that we will be able to continue rickettsial studies as well as to expand appropriate aspects of these studies. For the present time the grant is administered through the Department of Anatomy, Harvard Medical School.

Research Progress: During the course of this grant period a number of unforeseen changes in personnel in the professional and research assistant group has taken place. In spite of these events it is felt that we have made considerable progress on this contract.

In the early period of the current year, Dr. Michael Hollingdale completed his program of work on the antigens of Rickettsia quintana. An enzyme immunoassay (ELISA) test for detecting antibodies to R. quintana in sera from patients with trench fever and further evaluated, using the soluble antigen previously described. The test proved highly senstive and detected antibodies in several sera from known trench fever cases that were negative in other serological tests. However, some sera from other rickettsial infections also reacted. Nine of 9 scrub typhus sera were positive, as were several epidemic and endemic typhus sera, and a single Rocky Mountain spotted fever serum. The cross-reacting antigen appeared not to be cytoplasmic protein but a cell envelope lipopolysaccharide (LPS) antigen. This confirms the results of counterimmunoelectrophoresis (CIE) where cross reaction using solubilized cell envelope antigens occurred but no cross reactions occurred with soluble antigen. It is likely that the absence of cross reactions of the soluble antigen in CIE compares to ELIA is due to the lower sensitivity of the CIE test and the small amounts of LPS in the soluble antigen released by sonication (as reported earlier) that are reactive only in ELISA.

During this program, LPS has been demonstrated in R. quintana by various procedures, it is the first observation of rickettsial LPS. It appears serologically related to a similary component in R. tsutsugamushi (scrub typhus). The possibility exists in addition that rickettsial LPS is similar, if not identical, in all species. Numerous serological tests have been described, including passive hemagglutination, latex agglutionation, radioimmunoprecipitation, CIE and ELIA. Each test differs in sensitivity, specificity and ease of operation, with CIE being the most suitable for day-to-day use, particularly for large numbers of sera. Other antigens of R. quintana have been investigated and described. It is felt that these results are of considerable use and application to the other rickettsial species and for the elucidation of rickettsial pathogeneicity. The owl monkey experiments with Rochalimaea quintana were continued. At two-weekly intervals, the infected monkeys are bled and the blood screened for rickettsemia and serum antibody.

Stock supplies of Gilliam, Kato and Karp strains of scrub typhus were made by serial propagation in six day embryonated chicken eggs. Freshly harvested scrub typhus infected yolk sac was homogenised and used at a 10% concentration to infect cell cultures. Vero, BT_{20}, tick, and McCoy cells were infected and all cell lines supported rickettsial growth. However, McCoy cells provided the best results with a 4+ infection in 3 days. This cell system was used to propagate scrub typhus for attempts at soluble antigen preparation.

Guinea pigs were used to raise antisera against the yolk sac grown scrub typhus. The antigen was prepared by solubilizing with sonication followed by centrifugation. The soluble antigen was inoculated in guinea pig foot pads with Freunds complete adjuvant. The antisera acquired by this method was of such a low titer that no precipitin bands could be observed on immunodiffusion plates. However, fluorescent antibody (FA) techniques indicated a low level of antibody directed toward the scrub typhus rickettsia. After consulting with Ms. Marilyn Bozeman, we inoculated guinea pigs intra-cerebrally with the yolk sac grown antigen and collected antisera in 28 days.

To eliminate precipitin bands associated with yolk sac components, soluble antigen was prepared from scrub typhus agent grown in McCoy cells. The host cell was lysed by freezing and thawing; the resultant material was sonicated in the presence of 1% v/v Triton X-100 and partially clarified by centrifugation. The supernatant was concentrated 3-fold by dialysis against ethlene glycol. The final soluble antigen provided no detectable precipitin lines against the 28 day antisera. However, FA demonstrated antibody directed to scrub rickettsia even though at low titers. The soluble antigen was placed on an acrylamide gel electrophoretic system along with solubilized McCoy cells for a control. Only parallel bands were detected, indicating our rickettsial antigen is at a very low level. Therefore, we are now working at increasing the concentration of rickettsial antigen. Our first approach is to double the amount of infected McCoy cell monolayers and partially clarify the rickettsia by density gradient centrifugation according to Obijeski and Palmer (personal communication) using potassium tartrate and glycerol.

We will not be able to proceed with the two dimensional immunoelectrophoresis until we observe precipitin bands in the immunodiffusion plates. Precipitin bands are dependent on adequate levels of antigen and precipitating antibody. We do plan to look at proteins of the various antigen preparations in an effort to describe rickettsial specific protein bands by acrylamide gel electrophoresis. If we are successful in doing this, isoelectric focusing can be employed to further resolve scrub typhus proteins.

Throughout this period we have made routine fixations for electron microscopy of scrub typhus microorganisms gorwn in the McCoy cells and in some of the chick yolk sacs from infected embryos. Thin sections of embedded material were examined to confirm the Giemsa stained smears for the frequency and intracellular localization of the rickettsia. A series of electron micrographs of R. tsutsugamushi in infected cells has been made.

After the active assistance and collaboration of Dr. MacDonald was arranged for the present study, we have made much progress in our aim to grow rickettsia and isolate antigens. Concurrent with this effort, electron microscopy has been fully utilized to monitor and direct these efforts. Samples of many infected cultured cells, as well as pellets, remaining after extraction of the soluble antigens were examined in thin sections by transmission electron microscopy.

We have found that R. tsutsugamushi grows moderately well in BHK_{21} cells whether grown as a monolayer or in suspension cultures. The previous work on McCoy cells where these same rickettsiae could be grown in great abundance was discontinued because of the presence of numerous mycoplasma and C type virus particles normally present in this cell line. These were observed by electron microscopy.

On an attempt to induce rapid proliferation and larger numbers of rickettsiae, BHK_{21} cells were irradiated with 5,000 R from a cobalt source prior to inoculation with R. tsutsugamushi. This radiation inhibits cell division and proliferation of the BHK_{21} cells but does not kill them. Furthermore, this treatment does not seem to cause marked ultrastructural changes in the cultured cells but irregular shaped nuclei and multinucleated cells were common. In these irradiated cells the rickettsia grew in great abundance within days compared to the weeks required for a lower concentration of rickettsiae. The actual determination of the ultrastructural features of the infection and proliferation of R. tsutsugamushi in these irradiated cells remains to be determined but some preliminary observations on their distribution and characteristics have been observed.

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